The NS3 proteins of global strains of bluetongue virus evolve into regional topotypes through negative (purifying) selection

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Abstract

Comparison of the deduced amino acid sequences of the genes (S10) encoding the NS3 protein of 137 strains of bluetongue virus (BTV) from Africa, the Americas, Asia, Australia and the Mediterranean Basin showed limited variation. Common to all NS3 sequences were potential glycosylation sites at amino acid residues 63 and 150 and a cysteine at residue 137, whereas a cysteine at residue 181 was not conserved. The PPXY and PS/TAP late-domain motifs were conserved in all but three of the viruses. Phylogenetic analyses of these same sequences yielded two principal clades that grouped the viruses irrespective of their serotype or year of isolation (1900–2003). All viruses from Asia and Australia were grouped in one clade, whereas those from the other regions were present in both clades. Each clade segregated into distinct subclades that included viruses from single or multiple regions, and the S10 genes of some field viruses were identical to those of live-attenuated BTV vaccines. There was no evidence of positive selection on the S10 gene as assessed by reconstruction of ancestral codon states on the phylogeny, rather the functional constraints of the NS3 protein are expressed through substantial negative (purifying) selection.
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1. Introduction

Bluetongue (BT) is an insect-transmitted viral disease of ruminants that was first described in southern Africa (Spreull, 1905), and subsequently elsewhere in the world (Verwoerd and Erasmus, 2004; Enserink, 2006; MacLachlan and Osburn, 2006). BT virus (BTV) has now been isolated from ruminants and/or vector insects from all continents except Antarctica (Tabachnick, 2004). Because BTV infection of ruminants is not contagious, the global distribution of BTV coincides with the distribution of competent *Culicoides* insect vectors and appropriate climatic conditions (Meiswinkel et al., 2004; Mullens et al., 2004). Different species of *Culicoides* insects serve as the principal vectors of BTV in distinct global ecosystems (Gibbs and Greiner, 1994; Tabachnick, 2004). Fewer than 20 of >1000 *Culicoides* species that occur worldwide have been proven or incriminated as vectors of BTV, and the principal vector species differ between regions as do the serotypes of BTV contained therein (Meiswinkel et al., 2004; Tabachnick, 2004; Gomulski et al., 2006).

BTV is the prototype member of the genus *Orbivirus*, family Reoviridae (Mertens et al., 2004; Fauquet et al., 2005). The BTV genome consists of 10 segments of double-stranded RNA that collectively encode seven structural (VP 1–7) and four non-structural (NS1–3/3A) proteins. Serotype is determined by epitopes on outer capsid protein VP2 that are influenced by conformational interaction with VP5 (DeMaula et al., 2000), thus analysis of serotype alone does not address the marked genetic variation that occurs within each of the 24 BTV serotypes. BTV evolves through a combination of genetic drift and shift (Samal et al., 1987; Bonneau et al., 2001), the latter from reassortment of individual genes during mixed infections of either the insect or ruminant hosts. Phylogenetic analysis of the nucleotide (nt) sequence of the gene encoding the VP3 core protein of BTV strains from Australia, South Africa and the United States (US) first showed that cognate BTV genes may differ depending on the geographic origin of each virus, a process termed "topotyping" (Gould, 1987; Gould and Pritchard, 1990). Subsequent phylogenetic analyses of the S10 gene that encodes the NS3/3A proteins also reliably segregated BTV strains from Asia, Europe and the US into distinct topotypes (Bonneau et al., 1999; Pritchard et al., 2004; Nikolakaki et al., 2005), whereas relationships were ambiguous when S10 genes of virus strains from South Africa were included in the analysis (van Niekerk et al., 2003).

Given the apparent segregation of BTV strains into region-specific clusters we used statistical tests based on the phylogeny to determine if the evolution of NS3 is influenced by positive selection pressures imposed within each global ecosystem, an approach apparently not previously used. NS3 was selected for phylogenetic analysis because prior studies have concluded that it is useful for topotype analysis, a substantial amount of full-length S10 sequence data from strains of BTV from around the world was available, and it has previously been suggested that NS3 may exert an important role in determining the vector status of individual species of *Culicoides* insects for both BTV and related African horse sickness virus (Martin et al., 1998; Mertens et al., 2004; Breard et al., 2007). The S10 gene encodes 2 proteins; the 229 amino acid NS3 protein and the 216 amino acid NS3A protein whose synthesis is initiated from an in-frame downstream AUG codon. The NS3 protein is essential for both virus assembly and release from infected cells (Bansal et al., 1998; Han and Harty, 2004), and recent studies indicate that late-domain motifs in the amino terminal portion of NS3 assist virus release by recruiting cellular ESCRT-I protein Tsg101 (Wirblich et al., 2006).

2. Materials and methods

2.1. Viruses and sequencing

The entire coding sequence of the S10 genes of 137 strains of BTV identified between 1900 and 2003 from Africa, Asia (Indonesia, China, India), Australia, Caribbean Basin and Central America (Costa Rica, Dominican Republic, Guatemala, Honduras, Jamaica, Puerto Rico, Trinidad), Mediterranean Basin (Cyprus, Israel, Italy, France [Corsica], Greece), and US were obtained from GenBank or by direct RT-PCR amplification and sequencing of cell culture propagated strains of individual viruses, essentially as described (Table 1; Gould, 1988; Pierce et al., 1998; Bonneau et al., 1999, 2002; van Niekerk et al., 2003; Pritchard et al., 2004; Nikolakaki et al., 2005). The S10 genes of live-attenuated vaccine strains of BTV developed in South Africa including those introduced

Table 1
Strains of bluetongue virus

Country of origin	Year of isolation	Serotype	ID[a]	Country of origin	Year of isolation	Serotype	ID[a]
North America							
USA	1983	2	N2US230[b]	Italy	2002	2	N2IT4913
USA	1953	10	N10US372[c]	Tunisia	2000	2	N2TUN114
USA	1980	10	N10US379[c]	South Africa	1944	3	N3SA917[f]
USA	1980	10	N10US380[c]	Cyprus (South African vaccine)	1944	3	N3SAVAC918[f]
USA	1981	10	N10US381[c]	South Africa	1999	3	N3SA906[f]
USA	1981	10	N10US382[c]	South Africa (vaccine)	1900	4	N4SAVAC908[f]
USA	1990	10	N10US384[c]	Greece	1979	4	N4GR650[g]
USA	1990	10	N10US385[c]	Greece	1999	4	N4GR623[g]
USA (vaccine)	UA[t]	10	N10USVAC376[c]	Greece	1999	4	N4GR622[g]
USA	1962	11	N11US373[c]	Greece	1999	4	N4GR626[g]
USA	1979	11	N11US386[c]	Greece	1999	4	N4GR691[g]
USA	1980	11	N11US702[c]	Greece	1999	4	N4GR621[g]
USA	1981	11	N11US383[c]	Greece	1999	4	N4GR627[g]
USA	1981	11	N11US703[c]	Greece	1999	4	N4GR624[g]
USA	1981	11	N11US704[c]	Greece	1999	4	N4GR620[g]
USA (Vaccine)	UA	11	N11USVAC377[c]	Greece	1999	4	N4GR692[g]
USA	1967	13	N13US374[c]	Greece	1999	4	N4GR653[g]
USA	1979	13	N13US713[c]	Greece	1999	4	N4GR654[g]
USA	1980	13	N13US711[c]	Greece	1999	4	N4GR655[g]
USA	1981	13	N13US712[c]	Greece	1999	4	N4GR656[g]
USA	1989	13	N13US710[c]	Greece	1999	4	N4GR02
USA	1962	17	N17US375[c]	Israel	2001	4	N4ISR222
USA	1979	17	N17US706[c]	Israel	2001	4	N4ISR226
USA	1980	17	N17US705[c]	Italy	2003	4	N4IT10353
USA	1981	17	N17US707[c]	Italy	2003	4	N4IT9034
USA	1990	17	N17US708[c]	South Africa	1900	4	N4SA909[f]
USA	1990	17	N17US709[c]	South Africa	1999	4	N4SA905[f]
USA (vaccine)	UA	17	N17USVAC378[c]	South Africa	1999	4	N4SA907[f]
USA	1997	ND[i]	NXUS280[d]	South Africa (vaccine used in Italy).	2003[e]	4	N4SAVAC
USA	1997	ND[i]	NXUS286[d]	South Africa (vaccine)	1937	8	N8SA919[f]
USA	1997	ND[i]	NXUS287[d]	South Africa	1937	8	N8SA924[f]
USA	1997	ND[i]	NXUS288[d]	South Africa	1999	8	N8SA938[f]
USA	1997	ND[i]	NXUS289[d]	Greece	1998	9	N9GR625[g]
USA	1997	ND[i]	NXUS290[d]	Greece	1999	9	N9GR651[g]
USA	1997	ND[i]	NXUS291[d]	Italy	2000	9	N9IT217
USA	1997	ND[i]	NXUS292[d]	Italy (laboratory propagated vaccine strain from South Africa)	2001	9	N9IT034
USA	1997	ND[i]	NXUS293[d]	Greece	1999	9	N9GR02
USA	1997	ND[i]	NXUS294[d]	South Africa (vaccine used in Italy	2003[e]	9	N9SAVAC
USA	2000	ND[i]	NXUS295[d]	South Africa (vaccine)	1944	11	N11SAVAC922[f]
USA	2000	ND[i]	NXUS281[d]	South Africa	1944	11	N11SA923[f]
USA	2000	ND[i]	NXUS282[d]	South Africa	1999	11	N11SA921[f]
USA	2000	ND[i]	NXUS283[d]	Greece	1999	16	N16GR652[g]
USA	2000	ND[i]	NXUS284[d]	Israel	1999	16	N16ISR277
USA	2000	ND[i]	NXUS285[d]	Italy	2002	16	N16IT8054
				South Africa (vaccine used in Italy)	2003	16	N16SAVAC

Table 1 (*Continued*)

Country of origin	Year of isolation	Serotype	ID[a]	Country of origin	Year of isolation	Serotype	ID[a]
Central America and Caribbean Basin							
Honduras	1989	1	N1HON2172	South Africa	UA	18	N18SA915[f]
Costa Rica	1988	3	N3CRC2058	**Asia and Australia**			
Guatemala	1990	3	N3GTM2230	Australia	1979	1	N1AU253[h]
Trinidad	1989	3	N3TRD2165	China	1986	1	N1CH223[b]
Dominican Republic	1990	4	N4DRP2227	Indonesia	1990	1	N1ID049[j]
Honduras	1990	6	N6HON2187	Australia	1994	1	N1AU052[j]
Dominican Republic	1990	8	N8DRP2215	Australia	1996	1	N1AU057[j]
Jamaica	1988	12	N12JAM2016	India	1999	1	N1IN912[f]
Puerto Rico	1990	17	N17PRC283	India	1999	1	N1IN913[f]
Puerto Rico	1990	17	N17PRC298	China	1996	2	N2CH224[b]
				Indonesia	1990	3	N3ID050[j]
Africa and Mediterranean Basin							
South Africa (vaccine)	1958	1	N1SAVAC910[f]	China	1996	3	N3CH225[b]
South Africa	1958	1	N1SA911[f]	China	1996	4	N4CH226[b]
South Africa	1958	2	N2SA916[f]	China	1996	12	N12CH227[b]
Greece	2001	1	N1GR628[g]	China	1996	15	N15CH228[b]
Greece	2001	1	N1GR657[g]	China	1988	16	N16CH229[b]
South Africa	1999	2	N2SA920[f]	India	1999	18	N18IN914[f]
South Africa (vaccine)	UA	2	N2SAVAC094[f]	Australia	1995	20	N20AU055[j]
South Africa (vaccine used in Italy	2003	2	N2SAVAC	Australia	1995	20	N20AU057[j]
France	2000	2	N2COFR092	Indonesia	1990	21	N21ID059[j]
France	2001	2	N2COFR093	Australia	1994	21	N21AU053[j]
Italy	2000	2	N2IT8341	Australia	1995	21	N21AU054[j]
Italy (laboratory propagated vaccine virus from South Africa)	2001	2	N2IT033	Australia	1998	21	N21AU058[j]
Italy	2001	2	N2IT81640	Indonesia	1991	23	N23ID051[j]
Italy	2001	2	N2IT81641				
Italy	2002	2	N2IT6409				

[a] Gene Bank accession numbers in chronological order: North American BTV isolates: AF135230, AF044372, AF044379, AF044380–AF044385, AF044376, AF044373, AF044386, AF044702, AF044383, AF044703, AF044704, AF044377, AF044374, AF044713, AF044711, AF044712, AF044710, AF044375, AF044706, AF044705, AF044707, AF044708, AF044709, AF044378, AF397280, AF397286–AF397295, AF397281–AF397285; Central American and Caribbean BTV isolates: AY426598–AY426604, AY426595–AY426597; African and Mediterranean Basin BTV isolates: AF512910, AF512911, AF512916, AY677628, AY449657, AF512920, AF481094, AY775152, AF481092, AF481093, AY823220, AY438033, AY823221, AY823222, AY775154, AY775153, AF469114, AF512917, AF512918, AF512906, AF512908, AY449650, AY677623, AY677622, AY677626, AY691691, AY677621, AY677627, AY677624, AY677620, AY691692, AY449653–AY449656, AY775159, AY775157, AY775158, AY775155, AY775156, AF512909, AF512905, AF512907, AY775160, AF512919, AF512924, AY120938, AY677629, AY449651, AY775161, AY438034, AY823223, AY823224, AF512922, AF512923, AF512921, AF512915; Asian and Australian BTV isolates: D00253, AF135223, AF529049, AF529052, AF529057, AF512912, AF512913, AF135224, AF529050, AF135225–AF135229, AF512914, AF529055, AF529057, AF529059, AF529053, AF529054, AF529058, AF529051.

[b] Bonneau et al. (1999).
[c] Pierce et al. (1998).
[d] Bonneau et al. (2002).
[e] Unavailable.
[f] van Niekerk et al. (2003).
[g] Nikolakaki et al. (2005).
[h] Gould (1988).
[i] Not determined.
[j] Pritchard et al. (2004).

into the Mediterranean Basin were included (van Niekerk et al., 2003), as were those of similar vaccines from the US (Pierce et al., 1998).

2.2. Sequence and phylogenetic analyses

The inferred amino acid sequences of the S10 genes were aligned using default parameters of CLUSTAL X (Thompson et al., 1997), and the corresponding nt sequences were aligned based on the amino acid alignment. The S10 gene of epizootic hemorrhagic disease virus serotype 1 was used as the out-group to root the trees (Jensen and Wilson, 1995); previous analyses demonstrated that the S10 genes of BTV form a monophyletic group (Pierce et al., 1998; van Niekerk et al., 2003). Phylogenetic trees were inferred from nt data using unweighted maximum parsimony (MP). PAUPrat (Sikes and Lewis, 2001) was used to generate PAUP* commands to execute parsimony ratchet searches (Nixon, 1999) with PAUP* 4.0b10 (Swofford, 1998). Twenty independent parsimony ratchet searches were implemented, each with 200 ratchet iterations and perturbing 15% of the 256 parsimony-informative characters per replicate. Bootstrap MP searches employed 1000 pseudoreplicates, with each including

10 replicates of random taxon addition, a maximum trees setting of 10, and a search limit of 60 s. Testing for selection on individual codons was performed using the Datamonkey internet interface (Kosakovsky Pond and Frost, 2005a) to the HyPhy program (Kosakovsky Pond et al., 2005). The best-fit codon substitution model (TrN) and a neighbour-joining (NJ) tree were determined using HyPhy, and single-likelihood ancestor counting was used to assess the numbers of synonymous and non-synonymous substitutions for each codon site given the reconstruction of ancestral codon states on the NJ tree (Kosakovsky Pond and Frost, 2005b).

3. Results

3.1. Comparison of the NS3/3A proteins

Comparison of the deduced amino acid sequences of the S10 genes of 137 strains of BTV from Africa, the Americas, Asia, Australia and the Mediterranean Basin confirmed the conserved nature of the NS3/3A proteins, including potential glycosylation sites at amino acid residues 63 and 150, a cysteine at residue
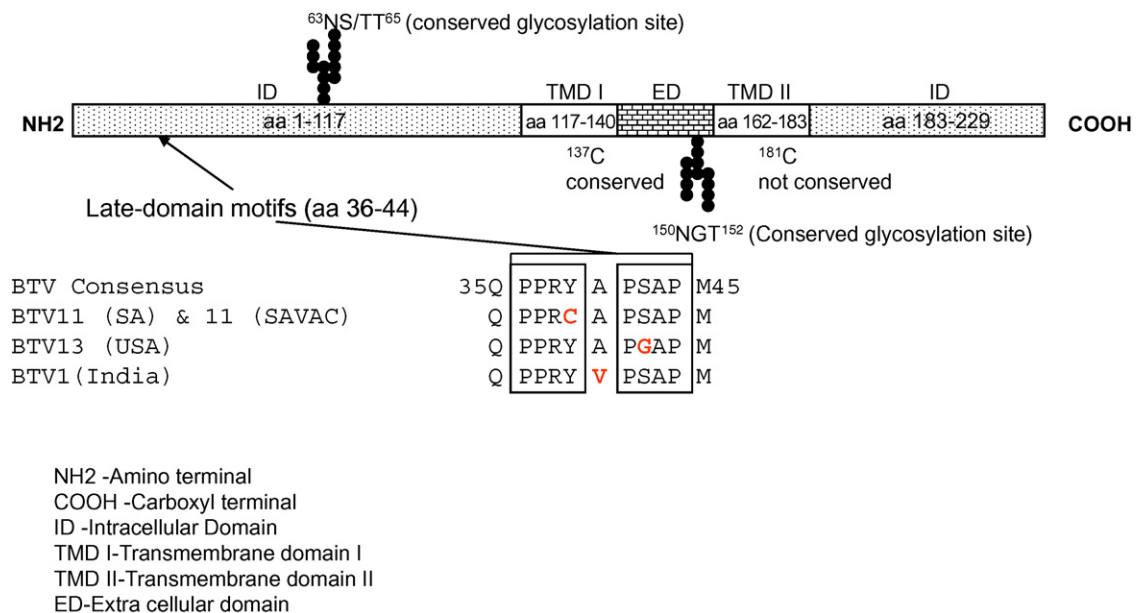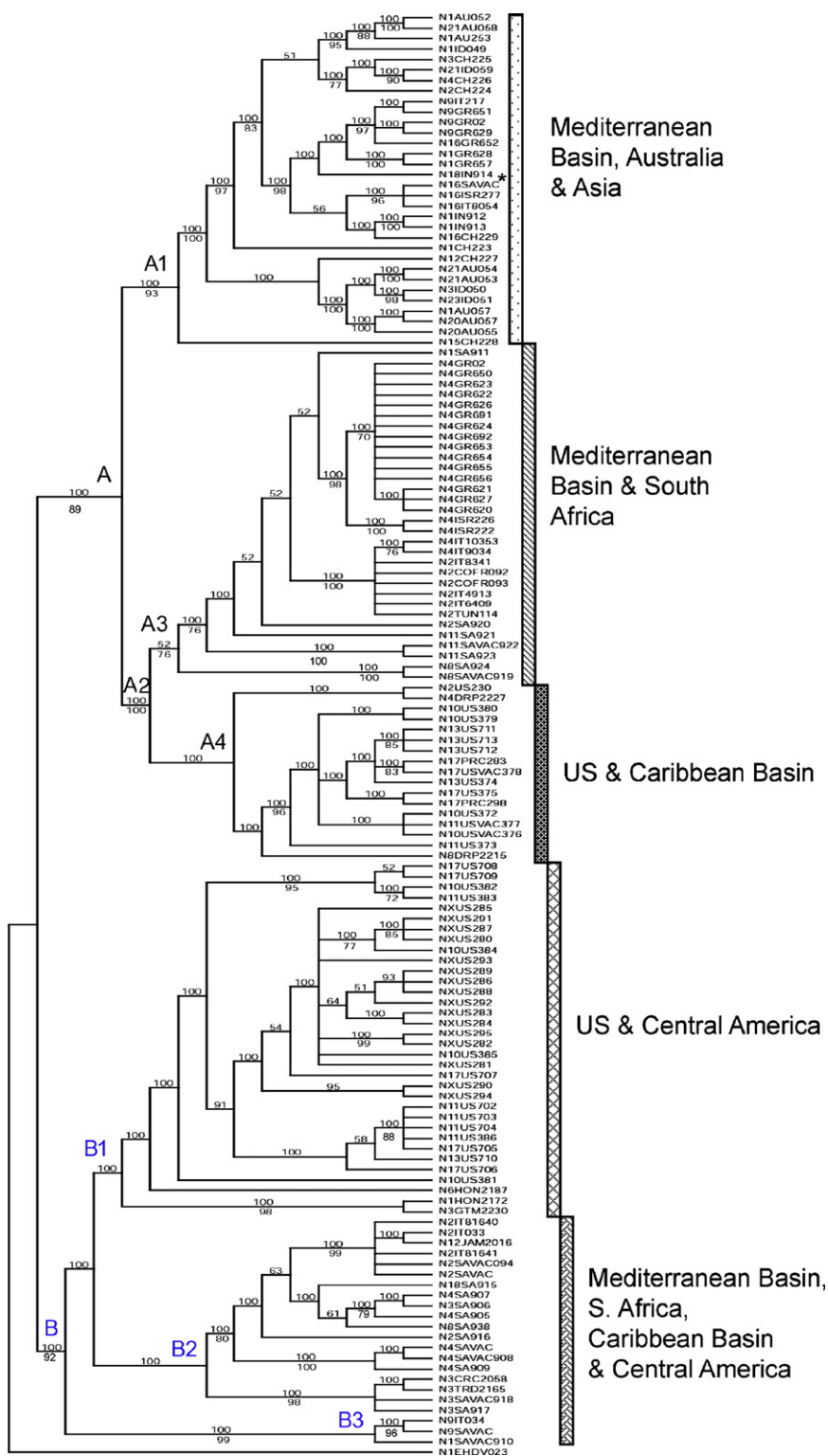

Fig. 1. Cartoon of BTV NS3 protein demonstrating essential features including transmembrane domains, glycosylation sites, cysteine residues, and the location and variation within the late-domain motifs of three strains of BTV and at residue 40 between these motifs in another.

137, and a tryptophan at residue 157 (Fig. 1). However, only 5 of 9 previously identified phenylalanine residues were conserved in all viruses, and 10 of 11 proline residues. There was also variation of the cysteine at residue 181. The PPXY and PS/TAP late-domain motifs located between residues 36 and 44 (Wirblich et al., 2006) were conserved in 134 of the 137 virus strains (Fig. 1). Two strains of serotype 11 had a Y to C substitution in the PPXY motif, and a strain of serotype 13 had an S to G substitution in the PS/TAP motif. A strain of BTV serotype 1 also had an A to V substitution at residue 40, which is located between the two motifs.

3.2. Phylogenetic analyses

Ratchet parsimony searches recovered 626 trees (Consistency Index = 0.41) of 1431 steps, and the majority-rule consensus tree is depicted in Fig. 2. Parsimony analysis yielded a large number of resolved clades by both strict and majority-rule consensus criteria. The most comprehensive clades (A and B in Fig. 2) were present in the strict consensus and also received high bootstrap support, and these two main clades grouped the different BTV strains irrespective of virus serotype or year of identification (Fig. 2; Table 1). Parsimony strict consensus results recovered distinct subclades within clades A and B; these subclades had different reliability as assessed by bootstrap resampling. Subclades A1, A2 and B3 had high bootstrap support, subclade A3 moderate support, and subclades A4, B1 and B2 were not reliably supported by bootstrap resampling (<70%).

The viruses from Asia (India, China, Indonesia) and Australia occur only in subclade A1, along with the South African vaccine strain of BTV 16, an Israeli isolate of serotype 16 (N16ISR277), and recent (since 1999) BTV isolates from Italy and Greece. Subclade A2 includes a geographically diverse collection of viruses, including a subclade (A3) representing viruses from the Mediterranean Basin (Italy, Corsica, Tunisia, Greece), Israel and South Africa. The sister

groups to the Mediterranean viruses in subclade A3 are South African viruses that include vaccine strains of BTV serotypes 8 and 11. Subclade A4 includes only viruses from the US and Caribbean Basin (Dominican Republic, Puerto Rico). All of the other viruses from the US are included in subclade B1, along with three viruses from Honduras and Guatemala that form sister groups to these US viruses. Subclade B2 includes a heterogeneous mixture of viruses from the Caribbean Basin (Jamaica, Costa Rica, Trinidad), Italy and South Africa (including vaccine strains of BTV serotypes 2–4, and the Italian vaccine strain of serotype 2 [N2IT033] derived from the South African virus), and subclade B3 includes South African vaccine strains of serotypes 1 and 9, and an Italian vaccine strain (N9IT034) derived from the South African vaccine strain of serotype 9.

There was no evidence of positive selection (adaptive molecular evolution) for any codon using a broad range of test P-values (0.01–0.20), which indicates that interaction between the NS3 protein and potential selection agents (e.g., different species of Culicoides vectors) does not result in detectable positive selection as determined by this approach. In contrast, a substantial number of codons (123 or 53% at P = 0.05) showed negative selection, consistent with strong functional constraints typical of purifying selection of the encoded NS3 protein. Unresolved, however, is the potentially confounding impact of the multiple vector species that occur within some individual ecosystems (Meiswinkel et al., 2004; Tabachnick, 2004; Savini et al., 2005; Gomulski et al., 2006) or changes that result from cell culture adaptation of many of the virus strains evaluated.

4. Discussion

Although the global distribution of BTV parallels that of competent insect vectors, different virus serotypes are disseminated by different insect vector species in distinct global ecosystems (Gibbs and

Fig. 2. Phylogenetic tree of NS3 genes of the strains of BTV. The tree represents the majority-rule consensus of 626 equally parsimonious trees inferred from ratchet analysis (699 characters, 256 parsimony-informative sites, 1431 steps, CI = 0.41). Parsimony consensus clade frequencies are shown above internal branches. Results from bootstrap resampling are shown below internal branches for groups occurring at >70% frequency in the bootstrap parsimony consensus tree. The NS3 gene of epizootic hemorrhagic disease virus serotype 1 (EHDV 1) was used as an outgroup to root the tree. (*) A vaccine strain of BTV16 that originally was derived from a virus isolated in Pakistan (N16SAVAC).

Greiner, 1994; Tabachnick, 2004; MacLachlan and Osburn, 2006). The current study confirms that the NS3 protein is highly conserved amongst global strains of BTV, even those from divergent ecosystems. The lack of exclusive monophyly within the phylogenetic tree (Fig. 2) for viruses of diverse geographic origin is consistent with recent movement of individual S10 genes between regions, such as the transient incursion of Indonesian strains of BTV into Australia (Pritchard et al., 2004). Similarly, a large group of recent BTV isolates from Italy, Corsica and Greece group in subclade A3 with a virus from Tunisia, consistent with the recent spread of BTV throughout the western Mediterranean Basin from North Africa and the fact that *Culicoides imicola* is the predominant but certainly not the sole vector in both regions (Purse et al., 2005; Gomulski et al., 2006). Another group of recent European strains of BTV, including several from Italy, Greece and Israel occur in subclade A1 with Asian strains of BTV, suggestive of an independent incursion into the Mediterranean Basin through the Middle East (Nikolakaki et al., 2005; Potgieter et al., 2005; Breard et al., 2007). The S10 genes of US viruses are also represented in both main clades, with subclades A4 and B1 including US and Central American (B1) or Caribbean (A4) strains that suggests a history of genetic continuity between the regions. These two subclades include all US strains but, importantly, no viruses from Africa, Europe, Asia or Australia. In distinct contrast to the other subclades, subclade B2 includes a geographically diverse array of viruses including strains from the Caribbean Basin, Central America, Italy and South Africa (including South African vaccine strains) that is not readily explained on the basis of current understanding of the global ecology of BTV infection (Tabachnick, 2004).

Data from the current study strongly suggest that live-attenuated BTV vaccines artificially can facilitate movement of viral genes between regions. Live-attenuated vaccines to BTV serotypes 2, 4, 9, and 16 that were produced in South Africa have been widely used to immunize ruminants in Italy and adjacent countries of Mediterranean Europe in an effort to control the recent BT epidemic (Gomez-Trejedor, 2004), and vaccine strains of BTV now circulate naturally in Italy (Ferrari et al., 2005). Furthermore, South African live-attenuated vaccines to BTV serotypes 2, 4, 6, 10, and 16 have been extensively

used in Israel for many years (Shimshony, 2004), and live-attenuated vaccines against BTV serotypes 3, 8, 9, 10, and 11 recently were introduced in Turkey and perhaps the Balkans (Panagiotatus, 2004). Interestingly, a 2002 Italian field strain of BTV (N16IT8054) that was isolated prior to the introduction of serotype 16 vaccine into Italy (in 2004) shares common ancestry with both an Israeli field isolate (N19ISR277) and the South African vaccine strain that originally was derived from a field isolate of BTV from Pakistan (subclade A1; Fig. 2), suggesting that the S10 gene of the strain of BTV serotype 16 that incurred into Italy might be derived from the vaccine used in Israel. Similarly, Italian field isolates of BTV serotype 2 (N2IT81640, N2IT81641) are closely related or identical to the South African vaccine strain of these serotypes (subclade B2; Fig. 2). The natural circulation of live-attenuated vaccine strains of BTV, or reassortment of genes from vaccine viruses with those of field strains, will clearly complicate the interpretation of future phylogenetic and molecular epidemiologic investigations of BTV infection in regions like the Mediterranean Basin where these vaccines have been introduced.

5. Conclusions

In summary, this extensive analysis confirms that there is very limited variation within the NS3 proteins of global strains of BTV. The S10 gene tree does not group BTV isolates according to serotype, or exclusively to geographic region; thus, the geographic structure of some subclades strongly suggests that reassortment must have occurred to account for the distribution of serotypes in these regions. These phylogenetic studies of the S10 gene also confirm and extend previous evidence for the recent spread of BTV strains into incursional areas such as the Mediterranean Basin, including those representing vaccine strains. The data further indicate that NS3 is not evolving by adaptive (positive) selection imposed by the different selection pressures (e.g., vector species) within each global ecosystem; rather the functional constraints on NS3 are expressed through typical negative (purifying) selection. BTV topotypes may represent evolutionary discontinuities from ancient geographic separation of virus strains with accumula-

tion of neutral changes in nt sequence over long periods of time. Alternatively, the entire spectrum of genetic diversity in individual BTV genes already exists in the virus strains that occur in the original endemic region of Africa so that each introduction into a new area was a founder event followed by viral expansion with the slow accumulation of neutral substitutions, which is consistent with the minimal variation reported to date amongst the S10 genes of field strains of BTV isolated during the current epidemic (1998–present) in the Mediterranean Basin (Nikolakaki et al., 2005; Breard et al., 2007). Thus, mechanisms such as genetic drift and founder effects (Bonneau et al., 2001), in combination with negative selection, are likely the most important factors governing the molecular evolution of the NS3 protein of field strains of BTV within each global ecosystem.

Acknowledgements

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